AAPS Interlaboratory Study

Tier 2 Method: Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)

Introduction

This protocol describes measurement of size distribution by sedimentation velocity analytical ultracentrifugation for samples diluted to within the linear absorbance range (0.2-1.2 O.D.) of the instrument. The results of the measurements are reported as relative abundances and sedimentation coefficients for each species present.

Important Notes: This protocol is not a Standard Operating Protocol and assumes the required instrument is in good working order and the analysis is performed by experienced user(s). Required calibration or check standards should be run according to the manufacturer instructions. This protocol does not contain all the details of the analysis. The analyst should rely on their best judgment, routine practices, and knowledge of the technique to conduct the study; this protocol should be used as a guideline for the analysis. Samples should be analyzed immediately after thawing and preparation.

Equipment and Materials List

- Beckman Coulter XL-A, XL-I, or Optima AUC
- An50Ti (8 hole) or An60Ti (4 hole) titanium rotor
- Assembled analytical sample cell
    a. Centerpiece
        i. 12 mm epon charcoal-filled centerpiece for low sample concentrations (customary)
    b. Window assembly
        i. Quartz or sapphire (recommended)
        ii. Liner
        iii. Window gasket
        iv. Window holder
    c. Cell housing
    d. Hex screws
    e. Neoprene gaskets
- Alignment tool
- Pipets and pipette tips
- Protein samples and formulation buffers supplied by sample originators (see Table 1)
    - Buffers, aliquots of unstressed, stir stressed, light stressed from Shipping #1

Reagents and Solutions

Table 1: The following samples and their buffers will be provided by sample originators. Buffers, aliquots of unstressed, stir stressed and light stressed from Shipping #1 should be used for this analysis. All stressed material were generated at nominally 1 mg/mL.

Proteins	Formulations	Samples
Amgen IgG2	10 mM sodium acetate, pH 5.0	Unstressed, 1 mg/mL Stir stressed, 1 mg/mL Light stressed, 1 mg/mL
NISTmAb IgG1	12.5 mM L-histidine, 12.5 mMol L-histidine HCl, pH 6.0	Unstressed, 1 mg/mL Stir stressed, 1 mg/mL Light stressed, 1 mg/mL

Procedure

1. Prior to sample preparation ensure the AUC chamber and rotor are equilibrated to the run temperature (20 °C). This will take approximately 1 hour.

2. Thaw the samples from Shipping #1 and aliquot the Tier 2 vials (for the unstressed, stir, and light stressed mAb samples) as described in the instructions into smaller aliquots for the various methods. Be sure to follow the procedure for vigorously mixing the sample prior to aliquoting.

3. For this analysis, thaw the aliquots designated for SV-AUC measurements (prepared and frozen in #1) by bringing them to room temperature for no more than 15 minutes.

4. Prior to analysis, mix the aliquot in the following manner: pipet with 1000 uL tip near the bottom of the sample tube 10 times in different directions but not touching the bottom or creating bubbles. Remove sample from near the bottom of the vial, from about ¾ of the depth of the tube from the top of the liquid before dispensing into the appropriate container for analysis.

5. If the sample is not transparent/clear visually, use adequate centrifugation to remove any suspended particles.

6. Prepare approximately 450-500 µL of sample for each replicate (three recommended) and have identical volumes of the appropriate buffer/diluent. For absorbance optics, a target O.D. of 0.5-0.8 is recommended, although O.D. of 0.2-1.2 are acceptable. For OD measurements, analysts can refer to the UV Visible Spectroscopy protocol.

7. Obtain a clean assembled AUC cell with a double sector centerpiece. Verify the assembled cell has been torqued to at least 120-inch pounds. For absorbance detection either sapphire windows or quartz windows may be used.
8. Load approximately 480 µL of buffer and sample into the appropriate sectors.
    a. For absorbance optics, it is recommended that approximately 20 µL less sample volume be loaded to offset the meniscus.

9. Seal the sector filling holes with a neoprene gasket and hex screw.
10. Obtain a pre-temperature equilibrated rotor with counterbalance. Verify a counterbalance weighs within ± 0.5 g of the sample cell directly across.
11. Ensure that the assembled cells have balanced weight such that the rotor will be balanced once all cells are loaded.
12. Load the assembled cells into the rotor and align cells to the center of rotation using an alignment tool.
13. Place the rotor into the AUC chamber and turn on the vacuum. Once the vacuum is below 100 µm Hg, allow the sample loaded rotor to equilibrate for at least 30 min.
14. If desired, a check at 3000 rpm may be performed prior to the actual run to verify proper sample loading and cell assembly. This is to check for proper sample absorbance and to ensure that none of the cells are leaking.
15. Instrument run parameters used are as follows:

Parameter	**Value**
Angular velocity (rpm)	40,000
Temperature (°C)	20
Detection (nm)	Absorbance at 280
R_{min} (cm)	5.8
R_{max} (cm)	7.05
Radial scan increment (cm)	0.003
Replicates	1
Mode	Continuous
Delayed start	0
Time between scans	4 min
Number of scans per cell	100

16. After the run is complete the cells must be removed and cleaned manually
    a. Disassemble cells and carefully place centerpiece and windows in a small plastic beaker with 1% Hellmanex to cover the materials. Warm the solutions in a microwave, then sonicate for 30 min.
    b. Remove centerpiece and windows from the beaker and rinse with copious water. Final rinse must be with high purity water (such as Milli-Q, HPLC grade).
    c. Dry centerpiece and windows.
    d. Reassemble the clean dry cell. Optionally add Spincote to the screw ring. Do not tighten the screw ring with the torque wrench at this time.

17. Data analysis is performed in SEDFIT v.16.1c software package using the following parameters:

Parameter	Value
Model	Continuous c(s)
Meniscus (from center of rotation) (cm)	Flot from max sample of side spike (cm)
Bottom (from center of rotation) (cm)	7.2 (fixed)
Top fitting limit (from center of rotation) (cm)	+0.1 from meniscus
Bottom fitting limit (from center of rotation) (cm)	7.0 or 6.7
Frictional ratio	1.6 (float)
Baseline	Float
RI noise	Disabled
TI noise	Enabled
Resolution	200, log spaced
Smin (S)	0.1
Smax (S)	40
Confidence level (F-ratio)	0.68
Scans included	~60 for full 8-hole rotor
Protein specific volume (mL/g)	0.73
Buffer density (g/cm^3)	1.00000
Buffer viscosity (P)	0.01002

Data should be entered in the provided SV-AUC template. If data was not obtained for any peaks listed on the templates, enter "NA" in the cells for that peak.

Most users will report c(s) distributions as generated by the data analysis software to be used as xy data for visual fitting.

Further Information

For any specific questions regarding this method, please contact Nikki Machalek at NMachalek@KBIBiopharma.com. Please copy aapsinterlab@nist.gov on your email.